

CONCEPTS OF PROBE DESIGN II. DESIGN OF TARGET-SPECIFIC PROBES

Authors: Willem J. Mulder, MSc, Geralda A.F. van Tilborg, MSc, Gustav J. Strijkers, PhD, and **Klaas Nicolay, PhD**

Affiliation: Biomedical NMR, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands

Introduction

Biomedical imaging plays a central role in clinical diagnostics, since it provides a wealth of information on the morphological, physiological and metabolic hallmarks of disease processes (1-3). The traditional use of diagnostic imaging, however, often depends on the detection of tissue changes that occur in a late stage of disease progression. The development of procedures, which allow the detection of disease in a more early stage, could therefore have a major impact in health care (4). Interventions might be arranged well before the development of severe symptoms, which will have a positive impact on disease outcome. Furthermore, the current diagnostic tools often lack specificity, *i.e.*, the disease-related alterations in tissue status might in principle result from several mechanisms. The differentiation between the possible molecular causes is of major importance, as it will have an impact on the interventions that are optimal to treat the disease and may also make a more individualized treatment possible. The more early detection of disease as well as the increased specificity of diagnostic procedures calls for sensitive and marker-specific imaging techniques. This is the domain of the rapidly expanding field of molecular imaging, in which new agents are combined with traditional imaging techniques to visualize biochemical pathways at the molecular and cellular level (2,5-7). Molecular imaging requires detailed knowledge of the molecular and cellular processes that are at the basis of the disease. Once a biochemical marker of the disease has been identified, a molecular imaging probe should be designed. The probe should be able to “home” to the marker of interest following delivery into the organism in order to allow for the indirect visualization of marker distribution with the imaging technique of choice. The present contribution deals with the technologies that are used to equip the molecular imaging probes with target specificity (8-10). Examples will be drawn from the field of molecular and cellular MR imaging. Nevertheless, many of the design criteria are also relevant for other imaging modalities.

MRI and traditional contrast agents

MRI yields high-resolution three-dimensional images of biological specimens. Its remarkable soft tissue contrast results from endogenous differences in water content, T_1 and T_2 relaxation times, and various other physicochemical properties of tissue water (11). The information content of MRI can be further enhanced by the use of exogenous contrast agents, including high-stability chelates of the Gd^{3+} -ion such as Gd-DTPA (12,13), the workhorse of traditional contrast-enhanced MRI. Such low-molecular weight Gd-based contrast agents are usually detected with dynamic T_1 -weighted MRI and provide important information on tissue status, including perfusion and vascular permeability. T_2 - and T_2^* -shortening agents that are most often based on crystalline FeO nano-particles (typically ranging from 10-50 nm in diameter) are also frequently used in biomedical research (14). These agents can be effectively detected with T_2 - or T_2^* -weighted MRI and they are among others used as blood pool agents, because of their prolonged blood plasma half lives. The above contrast agents possess no targeting moiety and therefore have limited value *per se* in target-specific MRI. Nevertheless, FeO particles have proven to be very useful for the measurement of macrophage activity in relation to inflammatory processes that play a key role in many diseases, including atherosclerosis (15,16) and multiple sclerosis (17). Activated macrophages appear to have a high capacity to internalize FeO particles, despite of the fact that the particles carry no specific macrophage-targeting devices.

MRI and target-specific contrast agents

The development of MR contrast agents (CA), which are targeted to specific molecular entities, could significantly enhance the utility of MR by combining the noninvasiveness and high spatial resolution of traditional MRI with the localization of specific molecular markers. Due to the inherently low sensitivity of MRI (in comparison with nuclear and optical imaging techniques), high local concentrations of the CA at the target site are required to generate significant changes in MR image intensity. To meet these requirements, the CA should associate to its biological target with high affinity and specificity. This is to a large extent determined by the nature of the targeting device. A CA that is conjugated with an antibody

directed against the biological marker of choice will usually achieve a higher binding specificity and affinity than a short peptide sequence. The targeted CA should have an as high as possible relaxivity. The relaxivities r_1 and r_2 of a CA (in units of $\text{mM}^{-1}\text{s}^{-1}$) define the efficiency by which the CA shortens the T_1 and T_2 relaxation times of water, respectively, as compared to the pre-contrast situation. The higher the relaxivity, the lower the minimal target concentration that can be reliably detected. In the case of a relatively high target concentration, the demand for a very high relaxivity is obviously less stringent. An example of that is the MRI-based visualization of fibrin, which is abundantly present in thrombi and can be detected with the use of fibrin-targeted peptides that are conjugated with 4 Gd-DTPA moieties (18-20).

For CA's that are to be used for T_1 -weighted MRI of sparse biological markers, there are two basic strategies to boost the relaxivity r_1 . First, the r_1 can be increased by conjugating a high number of Gd-chelates to a macromolecular scaffold, which can, for example, be based on dendrimers (21,22), or proteins (23), or by the use of nano-particles that can contain a high payload of Gd-chelates. An example of the latter are liposomes, which can be prepared in a typical size range from 50 to 250 nm and can accommodate up to several hundred thousand lipids with a Gd-containing polar head group (24-27). The above constructs can readily be equipped with optical (*e.g.*, fluorescent) labels for combined MRI and optical imaging studies. Secondly, the r_1 can be increased by increasing the rotation correlation time, τ_c , of the CA construct. The conjugation of the Gd-chelate to a higher generation dendrimer (21,22,28,29) or to a protein (23) leads to an increased effective τ_c of the CA as compared to the parent low-molecular weight Gd-chelate and this can result in an up to four-fold increase in the ionic r_1 (22). Smaller but still significant increases in r_1 have been observed for membrane-based CA nano-particles (25,27,30-32). The most popular and most effective T_2 - and T_2^* -shortening MRI CA's are based on nano-particles prepared from crystalline FeO. These have a very high r_2 and can be prepared in a range of sizes and with different coatings, depending on the application (14).

Apart from the relaxivity, the target specificity and affinity and the type of wanted contrast, many other criteria for the design of the CA constructs have to be considered. These include the pharmacokinetics, pharmacology and biological safety of the CA material, the location of the biological target (*e.g.*, intra-versus extravascular), the possible need for combining MRI-based molecular imaging with a complimentary technique for multi-modality imaging, or the wish to combine target-specific imaging with target-specific drug delivery. These aspects are beyond the scope of the present overview.

Introducing target specificity by linking ligands to MRI contrast agents

Several chemical conjugation strategies have been described to link a variety of targeting ligands to MRI contrast agents (33-35). These targeting ligands may include monoclonal antibodies (mAb), antibody fragments (Fab), (recombinant) proteins, peptides, peptidomimetics, sugars, and small molecules. Roughly, the ligand-CA coupling can be divided into non-covalent linkage and covalent linkage.

Non-covalent coupling is usually done with an avidin-biotin linkage. Avidin is a tetrameric protein with a molecular weight of 68 kDa and is capable of strongly binding 4 biotins ($K_A \approx 1.7 \times 10^{15} \text{ M}^{-1}$). The biotin-avidin interaction has been exploited for conjugating different types of MRI contrast agents with biotinylated proteins or peptides. For instance, conjugates of avidin and Gd-DTPA were used to target tumor cells pre labelled with biotinylated anti-HER-2/neu antibodies (36). Pre-formed conjugates of MRI nanoparticles and targeting ligands have also been used to *e.g.* magnetically label cells for MRI (37), as well as to actively target the nanoparticle to a specific receptor *in vivo*. The latter approach was used for the detection of the $\alpha_v\beta_3$ integrin on angiogenic blood vessels in tumor bearing rabbits (30). This non-covalent conjugation method is schematically depicted in Figure 1B. A nanoparticle equipped with several biotin molecules is first incubated with avidin. In a second step the particle-avidin conjugate is incubated with a biotinylated ligand, *e.g.* a peptide or an antibody. Although this method is simple and effective, the introduction of avidin in the conjugate has certain drawbacks. First, the size of the conjugate will increase by using avidin. More importantly, the immunogenic properties and the fast clearance of avidin by the liver have to be considered. In fact, this property of avidin can be used to an advantage to chase and clear antibodies (38) and MRI contrast agents from the circulation (23,39). Covalently linking the ligand to the contrast agent directly would lead to a smaller conjugate, which has more favorable pharmacokinetic properties. Several methods have been described. For dextran coated iron oxide particles amination can be achieved by incubating the nanoparticles with ammonia. Subsequently, these amines can be activated for conjugation to thiol (SH) exposing ligands, *e.g.* with SPDP (40,41) or SIA (42). Dextran-coated iron oxide nanoparticles conjugated to human holo-transferrin (Tf) have been used to image transgene expression in tumor bearing mice (43). Alternatively, maleimide containing entities, *e.g.* incorporated in lipidic

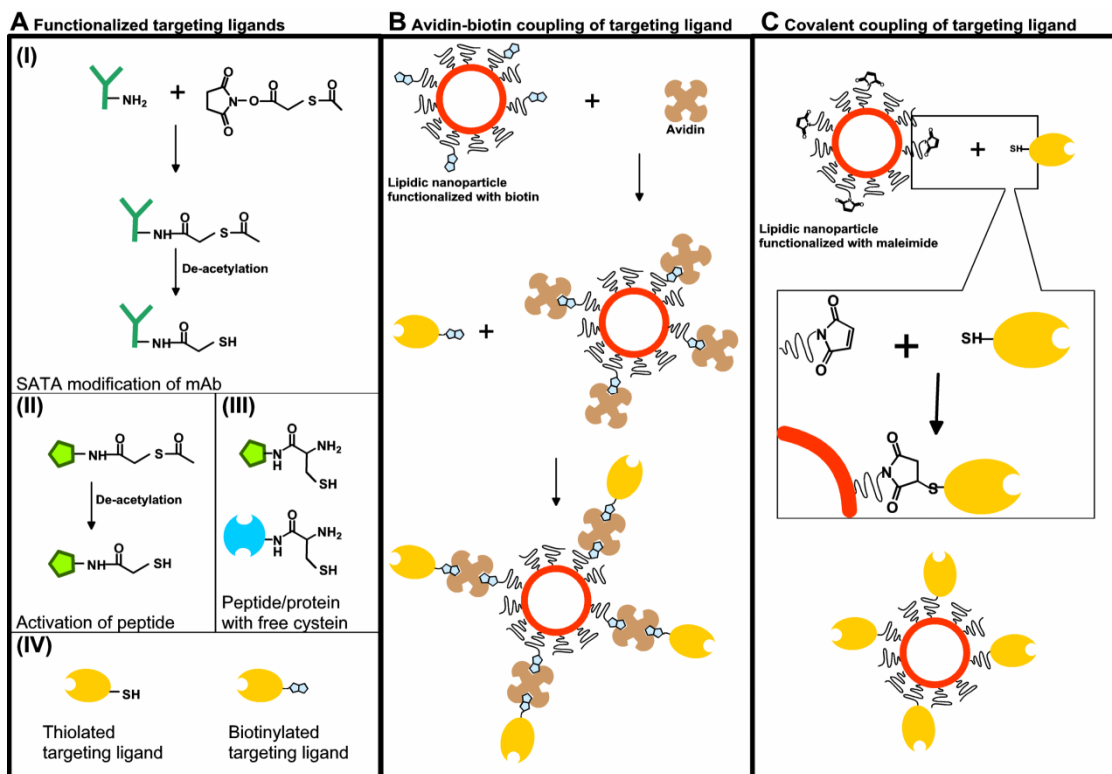


Figure 1. Strategies for the conjugation of targeting ligands to MRI contrast agents. (A) Introducing thiol-groups in (I) proteins or antibodies, and (II) peptides. (III) Protein and peptide with free cysteine. (IV) Targeting ligands with a functional group, i.e. thiol or biotin. (B) Schematic representation of avidin-biotin linkage of a ligand to a lipidic nanoparticle. (C) Schematic representation of maleimide-thiol linkage of a ligand to a lipidic nanoparticle.

nanoparticles, may be used to directly link thiol exposing ligands to the MRI contrast agent (25,26). Both techniques require the ligand to expose a thiol (SH), necessary for the bond formation. Proteins, antibody (fragments), and peptides exposing a free cysteine can directly be used for coupling to maleimide (Figure 1A III). Peptides, synthesized with a protective terminal thioacetate group, can be activated upon deacetylation with hydroxylamine (Figure 1A II). This results in the conversion of the thioacetate into a thiol group. The same strategy is used for proteins that are activated with succinimidyl-S-acetyl thioacetate (SATA) (Figure 1A I). SATA is coupled to free amine groups present in the protein, and with hydroxylamine the thioacetate moiety is converted into a free thiol group. The thiol-ligands react with maleimide containing particles and form a covalent thioether linkage, like schematically depicted in Figure 1C.

Other approaches that have been used to introduce target specificity are the incorporation of amphiphilic targeting proteins or peptides in the lipid bilayer of liposomes or the use of amphiphiles with a functional moiety, like a peptidomimetic in lipidic MRI nanoparticles (44,45). Furthermore, bioconjugates have been synthesized with small molecular ligands. Examples of such conjugates are Gd-DTPA-B(sLex)A, a Gd-DTPA based MRI contrast agent specific for the E-selectin receptor by conjugation to a Sialyl Lewis X mimetic (46) and conjugates of multimodal cyclic peptide-Gd(III)DTPA molecules equipped with either a fluorescent label (29) or biotin (21). Lastly, endogenous materials may be used as a CA vehicle. An example of such a MRI contrast agent is a bimodal probe based on high-density lipoprotein (HDL). HDL was made detectable for combined MRI and optical studies via the incorporation of paramagnetic and fluorescent lipids (47). A very elegant aspect of this HDL-based approach is that it does not require the synthesis and use of exogenous material, since isolated endogenous HDL may be used to create the contrast material.

Target specific contrast agents; the smart approach

Smart contrast agents, also referred to as responsive or activated contrast agents, are agents that undergo a large change in relaxivity upon activation. Such agents can be considered target specific in case they respond on e.g. transgene expression. A key publication in this field is from Louie et al. (48). These authors developed a Gd^{3+} chelating complex (EgadMe) which, in the presence of the enzyme β -galactosidase, undergoes a sizable increase in relaxivity. In its inactive form water is not accessible to Gd^{3+} because of blockage with a sugar moiety. When the enzyme cleaves off the sugar water can directly coordinate with Gd^{3+} explaining the r_1 increase. Peroxidase activity has been detected with MRI by using iron oxide nanoparticles conjugated with phenolic molecules that crosslink in the presence of peroxidases. This leads to the self-assembly of the nanoparticles (49), which results in a concentration dependent decrease of T_2 . A novel approach on smart MRI contrast agents uses endogenous iron to generate contrast. An adenoviral construct was used for the constitutive expression of human ferritin (50). The transfected cells become specifically superparamagnetic as they sequester endogenous iron from the organism. In vivo tests were done on mice, in which the virus was injected into the brains of the live animals. A strong MRI contrast at the site of injection was found within several days.

Conclusion

MRI is rapidly gaining importance as a molecular imaging technology, which adds a completely new dimension to the structural and functional information that the technique is renowned for. There are many ways, in which MRI contrast agents for specific biological targets can be prepared. The main challenge is to design effective contrast agents for intracellular targets.

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